Persistent epigenetic differences associated with prenatal exposure to famine in humans

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Extensive epidemiologic studies have suggested that adult disease risk is associated with adverse environmental conditions early in development. Although the mechanisms behind these relationships are unclear, an involvement of epigenetic dysregulation has been hypothesized. Here we show that individuals who were prenatally exposed to famine during the Dutch Hunger Winter in 1944–45 had, 6 decades later, less DNA methylation of the imprinted *IGF2* gene compared with their unexposed, same-sex siblings. The association was specific for periconceptional exposure, reinforcing that very early mammalian development is a crucial period for establishing and maintaining epigenetic marks. These data are the first to contribute empirical support for the hypothesis that early-life environmental conditions can cause epigenetic changes in humans that persist throughout life.

developmental origins | DNA methylation | insulin-like growth factor II | nutrition | periconception

Superimposed on the DNA sequence is a layer of epigenetic information that is heritable, particularly during mitosis, and controls the potential of a genomic region to be transcribed (1). Methyl groups coupled to cytosines in cytosine-guanine (CpG) dinucleotides and modifications of histones that package the DNA are the two main molecular marks that compose this information and regulate chromatin structure and DNA accessibility (2).

Animal studies have indicated that certain transient environmental influences can produce persistent changes in epigenetic marks that have life-long phenotypic consequences (3, 4). Early embryonic development is of special interest in this respect, because this is a crucial period for establishing and maintaining epigenetic marks (5). Indeed, culturing of preimplantation mice embryos found that epigenetic marks are susceptible to nutritional conditions in the very early stages of mammalian development (6, 7).

One of the rare opportunities for studying the relevance of such findings to humans is presented by individuals who were prenatally exposed to famine during the Dutch Hunger Winter (8). This period of famine was the consequence of a German-imposed food embargo in the western part of The Netherlands toward the end of World War II in the winter of 1944–45. During this period, registries and health care remained intact, so that individuals who were prenatally exposed to this famine can be traced. Moreover, the period of famine was clearly defined, and official food rations were documented. These unique features allow us to assess whether prenatal exposure to famine is associated with persistent epigenetic differences in humans.

One of the best-characterized epigenetically regulated loci is insulin-like growth factor II (*IGF2*). *IGF2* is a key factor in human growth and development and is maternally imprinted (9). Imprinting is maintained through the *IGF2* differentially methylated region (DMR), the hypomethylation of which leads to bi-allelic expression of *IGF2* (10). We recently studied *IGF2* DMR methylation in 372 twins (11). *IGF2* DMR methylation is a normally distributed quantitative trait that is largely determined by genetic factors in both adolescence and middle age, indicating that the methylation mark is stable up to middle age. Thus, if affected by environmental conditions early in human development, altered *IGF2* DMR methylation may be detected many years later.

Here we used our ongoing Hunger Winter Families Study (8) to investigate whether prenatal exposure to famine is associated with persistent differences in methylation of the *IGF2* DMR. Our primary focus was exposure during periconception, thus ensuring that the exposure was present during the very early stages of development that are critical in epigenetic programming. To further investigate the role of timing, we also studied individuals who were exposed late in gestation.

Results

Periconceptional Exposure. Our primary goal was to test whether periconceptional exposure to famine was associated with differences in *IGF2* DMR methylation in adulthood. Toward this end, we selected the 60 individuals from the Hunger Winter Families Study who were conceived during the famine 6 decades ago. The exposure period thus included the very early stages of development. The exposed individuals were compared with their same-sex sibling to achieve partial genetic matching. Using a quantitative mass spectrometry–based method (12, 13), the methylation of five CpG dinucleotides within the *IGF2* DMR was measured (11). Three CpG sites were measured individually, and two were measured simultaneously, because they could not be resolved due to their close proximity. All CpG sites but one were significantly less methylated among periconceptionally exposed individuals compared with their siblings ($1.5 \times 10^{-4} \leq P \leq 8.1 \times 10^{-3}$; see Table 1). The average methylation fraction of the *IGF2* DMR based on all five CpG sites was 0.488 among exposed siblings and 0.515 among unexposed siblings. Thus, periconceptional exposure was associated with a 5.2% lower methylation ($P = 5.9 \times 10^{-5}$), corresponding to 0.48 standard deviations (SDs) of the controls. The association was independent of sex ($P_{\text{interaction}} = 0.20$).

Fig. 1*A* displays the difference in *IGF2* DMR methylation within sibships according to the estimated conception date of the famine-exposed individual. *IGF2* DMR methylation was lowest in the famine-exposed individual among 72% (43/60) of sibships; this lower methylation was observed in conceptions across the famine period. Official daily rations were set weekly during the

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Table 1. *IGF2* DMR methylation among individuals periconceptionally exposed to famine and their unexposed, same-sex siblings

IGF2 DMR methylation	Mean methylation fraction (SD)				Relative change exposed	Difference in SDs	*P*
	Exposed (n = 60)		Controls (n = 60)				
Average	0.488	(0.047)	0.515	(0.055)	−5.2%	−0.48	5.9×10^{-5}
CpG 1	0.436	(0.037)	0.470	(0.041)	−6.9%	−0.78	1.5×10^{-4}
CpG 2 and 3	0.451	(0.033)	0.473	(0.055)	−4.7%	−0.41	8.1×10^{-3}
CpG 4	0.577	(0.114)	0.591	(0.112)	−2.3%	−0.12	.41
CpG 5	0.491	(0.061)	0.529	(0.068)	−7.2%	−0.56	1.4×10^{-3}

P values were obtained using a linear mixed model and adjusted for age.

famine period and were the same for every individual. The average daily rations were 667 kcal (SD, 151) (Fig. 1*A*), and there was little variation in the percentage of calories from proteins (≈12%, of which 4% of animal origin), fat (19%), and carbohydrates (69%) (14).

As a technical validation, *IGF2* DMR methylation was remeasured in 46 of 60 periconceptionally exposed individuals and their same-sex siblings, repeating the whole procedure from bisulfite treatment to quantification. A similarly lower 5.6% *IGF2* DMR methylation was observed ($P = 2.1 \times 10^{-3}$), confirming our initial findings.

Late Gestational Exposure. To further investigate the influence of timing, we selected the 62 individuals who were exposed to famine late in gestation for at least 10 weeks, so that they were born in or shortly after the famine. We found no difference in *IGF2* DMR methylation between the exposed individuals and their unexposed siblings (Table 2; Fig. 1*B*).

To formally test whether the association with lower *IGF2* DMR methylation depended on the timing of exposure, we analyzed the periconceptional and late exposure groups together with all 122 controls in a single model (Table 3). Periconceptional exposure was associated with lower methylation ($P = 1.5 \times 10^{-5}$), whereas late exposure was not ($P = 0.69$). Furthermore, there was statistically significant evidence for an interaction between timing and exposure ($P_{\text{interaction}} = 4.7 \times 10^{-3}$), indicating that the association was timing-specific.

Birth Weight. The mean birth weight of the 62 individuals exposed late in gestation was 3126 g (SD, 408), which is 296 g lower (95% confidence interval [CI], −420 to −170 g) than the mean (3422 g; SD, 464) of 324 reference births in 1943 at the same institutions ($P = 4 \times 10^{-6}$) (15). The lower birth weight underscores the impact of the famine during the Hunger Winter notwithstanding the absence of an association with *IGF2* DMR methylation. The mean birth weight of the 60 individuals who were exposed periconceptionally was 3612 g (SD, 648), not lower that that of the reference births (95% CI, +15 to + 365 g; $P = 0.03$). *IGF2* DMR methylation was not associated with birth weight ($P = 0.39$).

Age Association. To put the association of periconceptional famine exposure with a 5.2% lower *IGF2* DMR methylation into perspective, we assessed the relationship between age and *IGF2* DMR methylation in the 122 control individuals. Within the age range studied (43–70 years), a 10-year-older age was associated with a 3.6% lower methylation ($P = .015$).

Discussion

Here we report that periconceptional exposure to famine during the Dutch Hunger Winter is associated with lower methylation of the *IGF2* DMR 6 decades later. The hypomethylation that we observed is highly comparable to that found for the *Nr3c1* and *Ppara* genes in offspring of female rats fed an isocaloric protein-deficient diet starting before pregnancy (−8.2% and −10.2% vs −5.2% in our human study) (16), although greater effects for the *Agtr1b* gene have been found in a similar rat model (17). These data from animal models are consistent with the interpretation that famine underlies the *IGF2* hypomethylation that we observed and may be related to a deficiency in methyl donors, such as the amino acid methionine (3). An additional contribution of other stressors, such as cold and emotional stress (8), cannot be

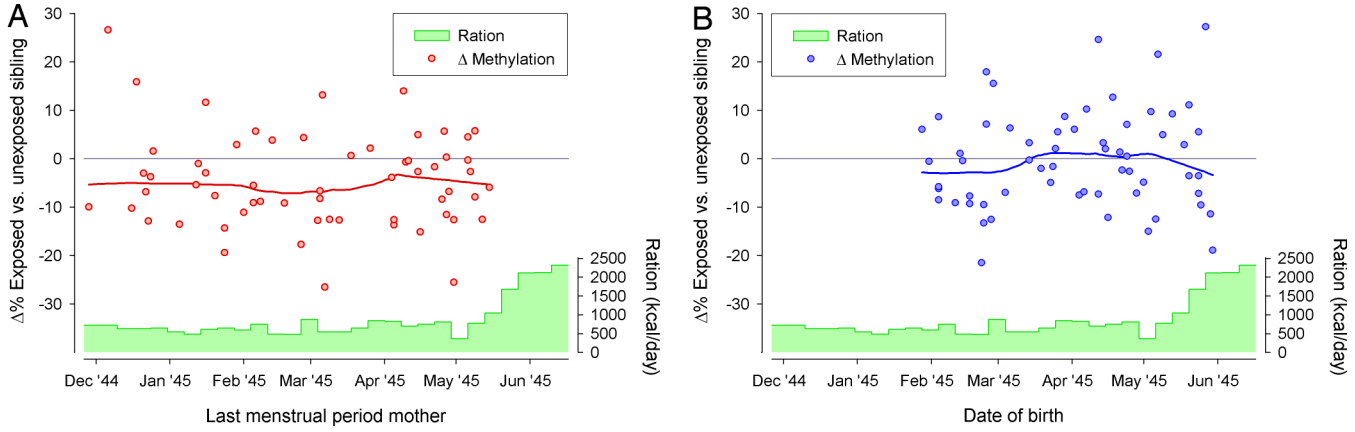

Fig. 1. Difference in *IGF2* DMR methylation between individuals prenatally exposed to famine and their same-sex sibling. (*A*) Periconceptional exposure: Difference in methylation according to the mother's last menstrual period (a common estimate of conception) before conception of the famine-exposed individual. (*B*) Exposure late in gestation: Difference in methylation according to the date of birth of the famine-exposed individual. To describe the difference in methylation according to estimated conception and birth dates, a lowess curve (red or blue) is drawn. The average distributed rations (in kcal/day) between December 1944 and June 1945 are depicted in green.

Table 2. *IGF2* DMR methylation among individuals exposed to famine late in gestation and their unexposed, same-sex siblings

IGF2 DMR methylation	Mean methylation fraction (SD)				Relative change exposed	Difference in SDs	*P*
	Exposed ($n = 62$)		Controls ($n = 62$)				
Average	0.514	0.045	0.519	0.036	−0.9%	−0.12	.64
CpG 1	0.460	0.044	0.464	0.048	−0.9%	−0.09	.68
CpG 2 and 3	0.462	0.039	0.471	0.039	−1.7%	−0.21	.46
CpG 4	0.602	0.085	0.612	0.073	−1.5%	−0.12	.30
CpG 5	0.529	0.060	0.531	0.060	−0.3%	−0.02	.77

P values were obtained using a linear mixed model and adjusted for age.

ruled out, however. Our study provides the first evidence that transient environmental conditions early in human gestation can be recorded as persistent changes in epigenetic information.

In contrast to periconceptional exposure to famine, exposure late in gestation was not associated with *IGF2* DMR methylation. Epigenetic marks may be particularly vulnerable during the very early stage of mammalian development, which is a crucial period for establishing and maintaining epigenetic marks (5). Experiments in which mouse zygotes were cultured to blastocysts favor this hypothesis (6, 7). The timing dependence of the association that we observed also may relate to the timing of tissue development, however (18). We studied blood and adult blood cells stem from the hematopoietic system, which is established relatively early in mammalian development (eg, day 10.5 in the mouse embryo [*cf.* weeks 4–6 in human gestation] (19)). Detailed future studies are needed to establish whether the susceptibility of epigenetic marks is an intrinsic property of early mammalian development or a general feature of newly developing tissues throughout gestation. Our results do not exclude the occurrence of epigenetic changes later in development (20) or during aging (21).

The developmental origins hypothesis states that adverse conditions during development contribute to adult disease risk (22). Although the mechanisms behind these relationships are unclear, the involvement of epigenetic dysregulation has been proposed (22–24). Our findings are a key element in elaborating this hypothesis. Human studies on the developmental origins of health and disease often use low birth weight as a proxy for a compromised prenatal development (22). Our data indicate that such studies are not necessarily sufficient for testing the involvement of epigenetics and thus extend our previous finding that birth weight is a poor surrogate for nutritional status during gestation (15). Epigenetic differences were found among individuals who were exposed to famine early in gestation and had a normal birth weight. Exposure to famine late in gestation was associated with low birth weight, as expected, but not with epigenetic changes. To monitor the crucial stages of early development, assessing maternal lifestyle, especially regarding nutrition (25), and embryo growth using three-dimensional ultrasonography (26) may be more appropriate than assessing birth weight.

The current study presents a first example of an association between a periconceptional exposure and DNA methylation in humans. It will be of prime interest to investigate whether other exposures during early development that are more common in modern societies, including overnutrition (3) and assisted reproductive technologies (27), give rise to similar associations. In addition, the extent to which epigenetic marks at other genomic regions are vulnerable to such exposures remains to be established. A key area to explore in future studies will be to assess the phenotypic consequences of changes in epigenetic marks. Diseases that have been associated with early gestational exposure to famine, such as schizophrenia (28) and coronary heart disease (29), are of particular interest in this respect. Analogous to current studies in genetic epidemiology (30), such epigenetic epidemiologic studies may need to be large and to include replication. Understanding how epigenetic control depends on early exposure may shed light on the link between development and health over the lifespan and ultimately suggest new ways to prevent human disease.

Methods

Study Population. The design of and recruitment for the Hunger Winter Families Study were described previously (8). Individuals exposed to famine prenatally were recruited by identification and follow-up of live singleton births in 1945 and early 1946 at three institutions in famine-exposed cities (the midwifery training schools in Amsterdam and Rotterdam and the Leiden University hospital). As controls, same-sex siblings and unrelated individuals from the same institutions who were born before or conceived after the famine period were recruited. Clinical examination, including blood sampling, was completed for 311 exposed individuals, 311 same-sex siblings, and 349 unrelated controls. Birth weight was abstracted from birth records from the three institutions. No birth weight data are available for the same-sex siblings who were not born at these institutions.

For the current epigenetic study, we focused on exposed individuals and their siblings as controls to achieve partial genetic matching in view of the

Table 3. Timing of famine exposure during gestation, *IGF2* DMR methylation, and birth weight

	Periconceptional exposure	Late gestational exposure	All controls
n	60	62	122
Males, %	46.7	45.2	45.9
Mean age, years	58.1 (SD, 0.35)	58.8 (SD, 0.4)	57.1 (SD, 5.5)
Birth weight, g	3612 (SD, 648)	3126 (SD, 408)	—
IGF2 DMR methylation			
Average	0.488 (SD, 0.047)	0.514 (SD, 0.045)	0.517 (SD, 0.047)
$P_{\text{vs all controls}}$	1.5×10^{-5}	.69	
$P_{\text{interaction}}$			4.7×10^{-3}

P values were obtained using a linear mixed model and adjusted for age.

high heritability of *IGF2* DMR methylation (11). From these, we selected the sibships with an individual exposed to famine periconceptionally and those with an individual exposed to famine late in gestation. Periconceptional exposure was defined as the mother's last menstrual period before conceiving the exposed individual between November 28, 1944 and May 15, 1945. This yielded 60 sibships. Exposure late in gestation was defined as a birth between January 28 and May 30, 1945, so that the duration of the famine exposure was at least 10 weeks. This yielded 62 sibships.

DNA Methylation. Methylation of the *IGF2* DMR was measured using genomic DNA from whole blood extracted using the salting-out method. One microgram of genomic DNA was bisulfite-treated using the EZ 96-DNA methylation kit (Zymo Research). Sibships were bisulfite-treated on the same plate. Three plates were used to process the 244 samples, each with an equal number of samples and a similar distribution in periconceptionally and late-exposed subjects. The region harboring the *IGF2* DMR (chr11:2,126,035–2,126,372 in NCBI build 36.1) was amplified using primers described elsewhere (11). DNA methylation was measured using a mass spectrometry–based method (Epityper, Sequenom) (12), the quantitative accuracy (R^2 duplicate measurements ≥ 0.98) and concordance with clonal polymerase chain reaction bisulfite sequencing of which has been reported previously (13, 31). All measurements were done in triplicate. CpG dinucleotides whose measurement was confounded by single nucleotide polymorphisms, as we discussed in a previous report (11), were discarded as part of quality control. The CpG dinucleotides reported in the current study were located at positions 41, 57 and 60, 202, and 251 bp in the amplicon targeting the *IGF2* DMR. Methylation data were 93% complete. DNA methylation of five CpG dinucleotides could be measured, three individually and two as a pair because they were directly adjacent and could not be resolved individually.

Statistical Analysis. The mean methylation fractions of individual CpGs and their SDs presented in the tables and figures are based on raw data. To obtain the average methylation of the whole *IGF2* DMR presented in the tables and figures, missing methylation data were first imputed using estimates from linear mixed models, thereby exploiting the correlations among CpG sites (11). To test for differences between exposed individuals and their unexposed siblings, age-adjusted linear mixed models were applied to the raw data without imputation of missing values. These analyses accounted for age at examination, family relations, correlated methylation of CpG dinucleotides, and methylation data missing at random. Exposure status, CpG dinucleotide, and age were entered as fixed effects, and sibship was entered as a random effect. The model including both the periconceptional and the late-exposure groups was extended with a variable indicating timing of the exposure and an interaction term of exposure status times exposure time. To test for the association between *IGF2* DMR methylation and birth weight, birth weight was added as a fixed effect. The linear mixed model may be viewed as an extension of the paired *t*-test; the model reduces to a paired *t*-test with identical outcomes if within-family methylation differences are assessed for a single CpG nucleotide and if data are complete and age adjustment is omitted. All *P* values are two-sided, and all statistical analyses were performed using SPSS 14.0.

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